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(57) Abstract

A method of combatting aberrant splicing in a pre-mRNA molecule containing a mutation is disclosed. When present in the pre-mRNA, the mutation causes the pre-mRNA to splice incorrectly and produce an aberrant mRNA or mRNA fragment different from the mRNA ordinarily encoded by the pre-mRNA. The method comprises hybridizing an antisense oligonucleotide to the pre-mRNA molecule to create a duplex molecule under conditions which permit splicing. The antisense oligonucleotide is one which does not activate RNase H, and is selected to block a member of the aberrant set of splice elements created by the mutation so that the native intron is removed by splicing and the first mRNA molecule encoding an native protein is produced. Oligonucleotides useful for carrying out the method are also disclosed.

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ANTISENSE OLIGONUCLEOTIDES WHICH COMBAT ABERRANT SPLICING AND METHODS OF USING THE SAME

This invention was made with government support under Grant No. GM32994 from the National Institutes of Health. The Government has certain rights to this invention.

Field of the Invention

The present invention relates to methods of combating aberrant splicing of pre-mRNA molecules and upregulating gene expression with antisense oligonucleotides, and antisense oligonucleotides useful for carrying out the same.

Background of the Invention

The potential of oligonucleotides as modulators of gene expression is currently under intense investigation. Most of the efforts are focused on inhibiting the expression of targeted genes such as oncogenes or viral genes. The oligonucleotides are directed either against RNA (antisense oligonucleotides) (M. Ghosh and J. Cohen, *Prog. Nucleic Acid Res. Mol. Biol.* **42**, 79 (1992); L. Neckers et al., *Crit. Rev. Oncog.* **3**, 175 (1992)) or against DNA where they form triplex structures inhibiting transcription by RNA polymerase II (J. Hanvey et al., *Science* **258**, 1481 (1992); W. McShan et al., *J. Biol. Chem.* **267**, 5712 (1992); M. Grigoriev et al., *J. Biol. Chem.* **267**, 3389 (1992); G. Duval-Valentin et al., *Proc. Natl. Acad. Sci. USA* **89**, 504 (1992)). To achieve a desired effect the oligonucleotides must promote a decay of the preexisting, undesirable protein by effectively preventing its formation de novo. Such techniques are not useful where the object is to upregulate production of the native protein. Yet, in cases where the

expression of a gene is downregulated because of mutations therein, a means for upregulating gene expression through antisense technology would be extremely useful.

Summary of the Invention

The present invention provides means for using antisense oligonucleotides to upregulate expression of a DNA containing a mutation which would otherwise lead to downregulation of that gene by aberrant splicing of the pre-mRNA it encodes.

Accordingly, a first aspect of the present invention is a method of combatting aberrant splicing in a pre-mRNA molecule containing a mutation. When present in the pre-mRNA, the mutation causes the pre-mRNA to splice incorrectly and produce an aberrant mRNA or mRNA fragment different from the mRNA ordinarily resulting from the pre-mRNA. More particularly, the pre-mRNA molecule contains: *(i)* a first set of splice elements defining a native intron which is removed by splicing when the mutation is absent to produce a first mRNA molecule encoding a native protein, and *(ii)* a second set of splice elements induced by the mutation which define an aberrant intron different from the native intron, which aberrant intron is removed by splicing when the mutation is present to produce an aberrant second mRNA molecule different from the first mRNA molecule. The method comprises hybridizing an antisense oligonucleotide to the pre-mRNA molecule to create a duplex molecule under conditions which permit splicing. The antisense oligonucleotide is one which does not activate RNase H, and is selected to block a member of the aberrant second set of splice elements so that the native intron is removed by splicing and the first mRNA molecule encoding a native protein is produced.

A second aspect of the present invention is a method of upregulating expression of a native protein in a cell containing a DNA encoding the native protein. Which DNA further contains a mutation which causes downregulation of the native protein by aberrant splicing thereof. More particularly, the DNA encodes a pre-mRNA, the pre-mRNA having the characteristics set forth above. The method comprises administering to the cell an antisense oligonucleotide having the characteristics described above so that the native intron is removed by splicing and the native protein is produced by the cell.

A third aspect of the present invention is an antisense oligonucleotide useful for combatting aberrant splicing in a pre-mRNA molecule containing a mutation. The pre-mRNA molecule contains a first set and second set of splice elements having the characteristics set forth above. The antisense oligonucleotide comprises an oligonucleotide which *(i)* hybridizes to the pre-mRNA to form a duplex molecule; *(ii)* does not activate RNase H; and *(iii)* blocks a member of the aberrant second set of splice elements.

The foregoing and other objects and aspects of the present invention are discussed in detail in the drawings herein and the specification set forth below.

Brief Description of the Drawings

Figure 1 shows the structure of pre-mRNAs. Boxes indicate exons; heavy lines, introns. Positions of the mutations (110 and 705) relative to nucleotide 1 of IVS 1 and IVS 2, respectively are shown above the HBΔ6 clone. Numbers below indicate the length, in nucleotides, of exons and introns. Antisense oligonucleotides are indicated by the numbered short bars below $ß^{110}$ and $IVS2^{705}$ constructs, and splicing pathways by the dashed lines.

Figure 2 shows the reversal of aberrant splicing by oligonucleotide 1 directed against the normal branch point in intron 1 of ß-globin pre-mRNA. The structure of the products and intermediates is depicted on the right; their size in nucleotides is shown on the left. An asterisk denotes aberrant mobility of lariat-containing intermediates. The same designations are used in the subsequent figures. Lane 1 shows splicing of control HBΔ6 pre-mRNA; lane 2 shows splicing of $ß^{110}$ pre-mRNA; lanes 3-8 show splicing of $ß^{110}$ pre-mRNA in the presence of increasing amounts (indicated at the top of the figure) of oligonucleotide 1; lane 9 shows splicing of $ß^{110}$ pre-mRNA in the presence of oligonucleotide 3, targeted to a sequence in intron 2 of ß-globin pre-mRNA.

Figure 3 shows the effects of oligonucleotide 2, directed against the aberrant 3' splice site in intron 1 of $ß^{110}$ pre-mRNA. Lane 1 shows splicing of $ß^{110}$ pre-mRNA; lanes 2-7 show splicing of $ß^{110}$ pre-mRNA in the presence of increasing amounts (indicated at the top of the figure) of oligonucleotide 2.

Figure 4 shows the reversal of aberrant splicing of $IVS2^{705}$ pre-mRNA by oligonucleotide 3 directed against the cryptic 3' splice site and oligonucleotide 4 directed against the aberrant 5' splice site in intron 2 of the $IVS2^{705}$ pre-mRNA. Lane 1 shows input RNA; lanes 2 and 3 show splicing of control transcripts (indicated at the top of the figure); lanes 4-8 and 9-13 show splicing of $IVS2^{705}$ pre-mRNA in the presence of oligonucleotide 3 and oligonucleotide 4, respectively. The amounts of the oligonucleotides in the reaction are indicated at the top. "?" on the left indicates apparent degradation product.

Figure 5 illustrates the effects of treating IVS2-654 cells with a 2'-O-methyl-ribooligonucleotide (17-mer) antisense oligonucleotide directed to a 3'

cryptic splice site in the presence of LIPOFECTIN™ transfection reagent. In lanes 1, 3 and 5, total cellulose RNA was subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out with primer A (specific for detecting aberrant splicing of the pre-mRNA); in lanes 2, 4 and 6, RT-PCR was carried out with primer C (specific for detecting correct splicing of pre-mRNA). Unspliced pre-mRNA and the correct and aberrant splice products thereof are schematically illustrated beneath the illustration of the gel lanes. Lanes 1 and 2 show splicing of pre-mRNA from cells treated with LIPOFECTIN™ transfection reagent and 3 μM pre-mRNA from 2'-O-methyl-oligoribonucleotide; lanes 3 and 4 show splicing of cells treated with LIPOFECTIN™ transfection reagent alone; lanes 5 and 6 show splicing of pre-mRNA from untreated cells.

Figure 6 is similar to Figure 5 above, and shows the effects of treating IVS2-654* cells with 5 and 20 μM antisense oligonucleotide directed to the 3' cryptic splice site in the presence of particles of replication deficient adenovirus. The RT-PCR reaction was carried out using primers that hybridize to the second and third exons of the human β-globin gene, respectively. Lane 1 shows splicing of pre-mRNA from untreated cells. Lanes 2 and 3 show splicing of pre-mRNA from cells treated with 5 and 20 μM oligonucleotide, respectively, in the presence of the adenovirus.

Figure 7 is similar to Figure 5 above, and shows the effects of electroporation of IVS2-654* cells treated with 5 or 50 μM of a 2'-O-methyl-ribooligonucleotide antisense oligonucleotide directed to an aberrant 5' splice site. Lane 1 shows splicing of pre-mRNA from untreated cells. Lanes 2 and 3 show splicing of pre-mRNA cells treated with 5 and 50 μM of the oligonucleotide, respectively.

Detailed Description of the Invention

Introns are portions of eukaryotic DNA which intervene between the coding portions, or "exons," of that DNA. Introns and exons are transcribed into RNA termed "primary transcript, precursor to mRNA" (or "pre-mRNA"). Introns must be removed from the pre-mRNA so that the native protein encoded by the exons can be produced (the term "native protein" as used herein refers to naturally occuring, wild type, or functional protein). The removal of introns from pre-mRNA and subsequent joining of the exons is carried out in the splicing process.

The splicing process is actually a series of reactions, mediated by splicing factors, which is carried out on RNA after transcription but before translation. Thus, a "pre-mRNA" is an RNA which contains both exons and intron(s), and an "mRNA" is an RNA in which the intron(s) have been removed and the exons joined together sequentially so that the protein may be translated therefrom by the ribosomes.

Introns are defined by a set of "splice elements" which are relatively short, conserved RNA segments which bind the various splicing factors which carry out the splicing reactions. Thus, each intron is defined by a 5' splice site, a 3' splice site, and a branch point situated therebetween. These splice elements are "blocked", as discussed herein, when an antisense oligonucleotide either fully or partially overlaps the element, or binds to the pre-mRNA at a position sufficiently close to the element to disrupt the binding and function of the splicing factors which would ordinarily mediate the particular splicing reaction which occurs at that element (e.g., binds to the pre-mRNA at a position within 3, 6, 9, 12, 15, or 18 nucleotides of the element to be blocked).

The mutation in the native DNA and pre-mRNA may be either a substitution mutation or a deletion

mutation which creates a new, aberrant, splice element. The aberrant splice element is thus one member of a set of aberrant splice elements which define an aberrant intron. The remaining members of the aberrant set of splice elements may also be members of the set of splice elements which define the native intron. For example, if the mutation creates a new, aberrant 3' splice site which is both upstream from (i.e., 5' to) the native 3' splice site and downstream from (i.e., 3' to) the native branch point, then the native 5' splice site and the native branch point may serve as members of both the native set of splice elements and the aberrant set of splice elements. In other situations, the mutation may cause native regions of the RNA which are normally dormant, or play no role as splicing elements, to become activated and serve as splicing elements. Such elements are referred to as "cryptic" elements. For example, if the mutation creates a new aberrant mutated 3' splice site which is situated between the native 3' splice site and the native branch point, it may activate a cryptic branch point between the aberrant mutated 3' splice site and the native branch point. In other situations, a mutation may create an additional, aberrant 5' splice site which is situated between the native branch point and the native 5' splice site and may further activate a cryptic 3' splice site and a cryptic branch point sequentially upstream from the aberrant mutated 5' splice site. In this situation, the native intron becomes divided into two aberrant introns, with a new exon situated therebetween. Further, in some situations where a native splice element (particularly a branch point) is also a member of the set of aberrant splice elements, it can be possible to block the native element and activate a cryptic element (i.e., a cryptic branch point) which will recruit the remaining members of the native set of splice elements to force correct splicing

over incorrect splicing. Note further that, when a cryptic splice element is activated, it may be situated in either the intron or one of the adjacent exons. Thus, depending on the set of aberrant splice elements created by the particular mutation, the antisense oligonucleotide may be synthesized to block a variety of different splice elements to carry out the instant invention: it may block a mutated element, a cryptic element, or a native element; it may block a 5' splice site, a 3' splice site, or a branch point. In general, it will not block a splice element which also defines the native intron, of course taking into account the situation where blocking a native splice element activates a cryptic element which then serves as a surrogate member of the native set of splice elements and participates in correct splicing, as discussed above.

The length of the antisense oligonucleotide (i.e., the number of nucleotides therein) is not critical so long as it binds selectively to the intended location, and can be determined in accordance with routine procedures. In general, the antisense oligonucleotide will be from 8, 10 or 12 nucleotides in length up to 20, 30, or 50 nucleotides in length.

Antisense oligonucleotides which do not activate RNase H can be made in accordance with known techniques. *See, e.g.*, U.S. Patent No. 5,149,797 to Pederson et al. (The disclosures of all patent references cited herein are to be incorporated herein by reference). Such antisense oligonucleotides, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligonucleotide as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the portions of the oligonucleotide involved in

duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous antisense oligonucleotides which do not activate RNase H are available. For example, such antisense oligonucleotides may be oligonucleotides wherein at least one, or all, of the internucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphonothioates, phosphoromorpholidates, phosphoropiperazidates and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described. In another non-limiting example, such antisense oligonucleotides are oligonucleotides wherein at least one, or all, of the nucleotides contain a 2' loweralkyl moiety (e.g., C1-C4, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). For example, every other one of the nucleotides may be modified as described. *See also* P. Furdon et al., *Nucleic Acids Res.* **17**, 9193-9204 (1989); S. Agrawal et al., *Proc. Natl. Acad. Sci. USA* **87**, 1401-1405 (1990); C. Baker et al., *Nucleic Acids Res.* **18**, 3537-3543 (1990); B. Sproat et al., *Nucleic Acids Res.* **17**, 3373-3386 (1989); R. Walder and J. Walder, *Proc. Natl. Acad. Sci. USA* **85**, 5011-5015 (1988).

The methods, oligonucleotides and formulations of the present invention have a variety of uses. They are useful in any fermentation process where it is desired to have a means for downregulating expression of a gene to be expressed until a certain time, after which it is desired to upregulate gene expression (*e.g.*, downregulate during the growth phase of the fermentation and upregulate during the production phase of the fermentation). For such use, the gene to be expressed may be any gene encoding a protein to be produced by fermentation so long as the

gene contains a native intron. The gene may then be mutated by any suitable means, such as site-specific mutagenesis (see T. Kunkel, U.S. Patent No. 4,873,192) to deliberately create an aberrant second set of splice elements which define an aberrant intron which substantially downregulates expression of the gene. The gene may then be inserted into a suitable expression vector and the expression vector inserted into a host cell (e.g., a eukaryotic cell such as a yeast, insect, or mammalian cell (e.g., human, rat)) by standard recombinant techniques. The host cell is then grown in culture by standard fermentative techniques. When it is desired to upregulate expression of the mutated gene, an antisense oligonucleotide, in a suitable formulation, which binds to a member of the aberrant second set of splice elements, is then added to the culture medium so that expression of the gene is upregulated.

The methods, oligonucleotides and formulations of the present invention are also useful as *in vitro* or *in vivo* tools to examine splicing in human or animal genes which are developmentally and/or tissue regulated. Such experiments may be carried out by the procedures described hereinbelow, or modification thereof which will be apparent to skilled persons.

The methods, oligonucleotides and formulations of the present invention are also useful as therapeutic agents in the treatment of disease involving aberrant splicing, such as ß-thalassemia (wherein the oligonucleotide would bind to ß-globin, particularly human, pre-mRNA), α-thalassemia (wherein the oligonucleotide would bind to α-globin pre-mRNA), Tay-Sachs syndrome (wherein the oligonucleotide would bind to β-hexoseaminidase α-subunit pre-mRNA), phenylketonuria (wherein the oligonucleotide would bind to phenylalanine hydroxylase pre-mRNA) and certain

forms of cystic fibrosis (wherein the oligonucleotide would bind the cystic fibrosis gene pre-mRNA), in which mutations leading to aberrant splicing of pre-mRNA have been identified (*See, e.g.*, S. Akli et al., *J. Biol. Chem.* **265**, 7324 (1990); B. Dworniczak et al., *Genomics* **11**, 242 (1991); L-C. Tsui, *Trends in Genet.* **8**, 392 (1992)).

Examples of β-thalassemia which may be treated by the present invention include, but are not limited to, those of the β^{110}, IVS1^{5}, IVS1^{6}, IVS2^{654}, IVS2^{705}, and IVS2^{745} mutant class (i.e., wherein the β-globin pre-mRNA carries the aforesaid mutations).

The term "antisense oligonucleotide" includes the physiologically and pharmaceutically acceptable salts thereof: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto. Examples of such salts are (a) salts formed with cations such as sodium, potassium, NH_4^+, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

Formulations of the present invention comprise the antisense oligonucleotide in a physiologically or pharmaceutically acceptable carrier, such as an aqueous carrier. Thus, formulations for

use in the present invention include, but are not limited to, those suitable for parenteral administration, including subcutaneous, intradermal, intramuscular, intravenous and intraarterial administration, as well as topical administration (i.e., administration of an aerosolized formulation of respirable particles to the lungs of a patient afflicted with cystic fibrosis). The formulations may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art. The most suitable route of administration in any given case may depend upon the subject, the nature and severity of the condition being treated, and the particular active compound which is being used.

The present invention provides for the use of antisense oligonucleotides having the characteristics set forth above for the preparation of a medicament for upregulating gene expression in a patient afflicted with an aberrant splicing disorder, as discussed above. In the manufacture of a medicament according to the invention, the antisense oligonucleotide is typically admixed with, *inter alia*, an acceptable carrier. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the patient. The carrier may be a solid or a liquid. One or more antisense oligonucleotides may be incorporated in the formulations of the invention, which may be prepared by any of the well known techniques of pharmacy consisting essentially of admixing the components, optionally including one or more accessory therapeutic ingredients.

Formulations of the present invention may comprise sterile aqueous and non-aqueous injection solutions of the active compound, which preparations are preferably isotonic with the blood of intended recipient and essentially pyrogen free. These

preparations may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions may include suspending agents and thickening agents. The formulations may be presented in unit dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

In the formulation the antisense oligonucleotide may be contained within a lipid particle or vesicle, such as a liposome or microcrystal, which may be suitable for parenteral administration. The particles may be of any suitable structure, such as unilamellar or plurilamellar, so long as the antisense oligonucleotide is contained therein. Positively charged lipids such as N-[1-(2,3-dioleoyloxi)propyl]-N,N,N-trimethyl-amoniummethylsulfate, or "DOTAP," are particularly preferred for such particles and vesicles. The preparation of such lipid particles is well known. *See, e.g.*, U.S. Patents Nos. 4,880,635 to Janoff et al.; 4,906,477 to Kurono et al.; 4,911,928 to Wallach; 4,917,951 to Wallach; 4,920,016 to Allen et al.;4,921,757 to Wheatley et al.; etc.

The dosage of the antisense oligonucleotide administered will depend upon the particular method being carried out, and when it is being administered to a subject, will depend on the disease, the condition of the subject, the particular formulation, the route of administration, etc. In general, intracellular concentrations of the oligonucleotide of from .05 to 50 μM, or more particularly .2 to 5 μM, are desired. For administration to a subject such as a human, a dosage

of from about .01, .1, or 1 mg/Kg up to 50, 100, or 150 mg/Kg is employed.

The present invention is explained in greater detail in the following non-limiting examples. Nucleotide sequences are presented herein by single strand only, in the 5′ to 3′ direction, from left to right.

EXAMPLE 1

Structure and Construction of Pre-mRNAs

The construction and structure of various human β-globin pre-mRNA molecules is illustrated in **Figure 1**. Boxes indicate exons; heavy lines, introns. Positions of the mutations (110 and 705) relative to nucleotide 1 of IVS 1 and IVS 2, respectively are shown above the HBΔ6 clone. Numbers below indicate the length, in nucleotides, of exons and introns. Antisense oligonucleotides (discussed in detail below) are indicated by the numbered short bars below $ß^{110}$ and $IVS2^{705}$ constructs, and splicing pathways by the dashed lines. All pre-mRNAs were transcribed by SP6 RNA polymerase (M. Konarska et al., *Cell* **38**, 731 (1984)) from appropriate fragments of human ß-globin gene subcloned into the SP64 vector. HBΔ6 (A. Krainer et al., *Cell* **36**, 993 (1984)) contains the whole human ß-globin gene. The ^{110} construct contains exons 1 and 2 and was subcloned from the original thalassemic clone (R. Spritz et al., *Proc. Natl. Acad. Sci. USA* **78**, 2455 (1981)). Before transcription, plasmids were linearized at the BamHI site. To construct the $IVS2^{705}$ plasmid, a fragment of HBΔ6 containing virtually the entire second exon, the entire second intron, and a major portion of the third exon was first subcloned in SP64 and subsequently subjected to site specific mutagenesis in accordance with known techniques (T. Kunkel et al., *Methods Enzymol.* **154**, 367 (1987)) to introduce a T to G mutation at nucleotide 705 of the

intron. Transcription was then carried out on a plasmid linearized at the PvuII site.

EXAMPLE 2

Synthesis of Antisense 2'O-methyl-Oligoribonucleotides

2'-O-methyl-Oligoribonucleotides for use in the examples described herein were synthesized in accordance with known techniques using reagents from Glen Research (Sterling, VA) and purified in accordance with known techniques using the SUREPURE™ purification kit available from US Biochemicals.

2'-O-methyl-oligoribonucleotides produced were referred to as oligo 1 through oligo 5.

Oligo 1 (GUCAGUGCCUAUCA) (**SEQ ID NO:1**), complementary to nucleotides 82-95 of intron 1, is targeted against the normal branch point, and oligo 2 (AUAGACUAAUAGGC) (**SEQ ID NO:2**), complementary to nucleotides 103-116 of intron 1, against the aberrant 3' splice site created by $ß^{110}$ mutation in intron 1 of the ß-globin gene. Oligo 3 (CAUUAUUGCCCUGAAAG) (**SEQ ID NO:3**), complementary to nucleotides 573-589 of intron 2, is targeted against the cryptic 3' splice site at nucleotide 579 of the second intron and oligo 4 (CCUCUUACCUCAGUUAC) (**SEQ ID NO:4**), complementary to nucleotides 697-713, is targeted against the aberrant 5' splice site created by the mutation at nucleotide 705 in the second intron of $IVS2^{705}$ pre-mRNA. Oligo 5 (GCUAUUACCUUAACCCAG) (**SEQ ID NO:5**) is targeted against the aberrant 5' splice site created by the $IVS2^{654}$ mutation (nucleotides 643-660 of intron 2). Oligo 6 (GCCUGACCACCAAC) (**SEQ ID NO:6**) is targeted against the cryptic 5' splice site in exon 1of globin pre-mRNA (nucleotides -23 to -10 relative to nucleotide 1 of intron 1).

EXAMPLE 3

Reversal of Aberrant Splicing by an Antisense Oligonucleotide Targeted Against the Normal Branch Point of Human β-Globin Intron 1

In $ß^{110}$- thalassemia, a form of the disease predominant in patients of Greek and Cypriot origin, an A to G mutation at nucleotide 110 of the first intron of human ß-globin gene creates an additional, aberrant 3' splice site (R. Spritz et al., *Proc. Natl. Acad. Sci. USA* **78**, 2455 (1981)). In spite of the presence of the normal 3' splice site, the aberrant site is preferentially used by the splicing machinery, resulting in an incorrectly spliced mRNA that contains 19 nucleotides of the intron sequence (**Fig. 1**). In cells transfected with $ß^{110}$-globin allele (M. Busslinger et al., *Cell* **27**, 289 (1981); Y. Fukumaki et al., *Cell* **28**, 585 (1982)) or during splicing of its transcript in nuclear extracts (R. Reed and T. Maniatis, *Cell* **41**, 95 (1985)) (see also Fig. 2, lane 2) correctly spliced mRNA constitutes only about 10% of the spliced product, consistent with the markedly reduced levels of normal hemoglobin observed in patients with this form of thalassemia. It was found that in $ß^{110}$ pre-mRNA the aberrant 3' splice site recruits the normal branch point at nucleotide 93 of the intron, competing with the correct 3' splice site, and thereby prevents correct splicing (R. Reed and T. Maniatis, *Cell* **41**, 95 (1985)). Significantly for this work, mutations inactivating the normal branch point activate a cryptic branch point at nucleotide 107 and result in splicing at the correct 3' splice site (Y. Zhuang and A. Weiner, *Genes and Dev.* 3, 1545 (1989)). Aberrant splicing cannot proceed due to the proximity of the cryptic branch point to the mutated 3' splice site at position 110.

To test whether antisense oligonucleotides targeted against the normal branch point sequence would

force the splicing machinery to select the cryptic branch point and generate a correctly spliced mRNA, a 14 nucleotide long 2'-O-methyl-oligonucleotide (oligonucleotide 1, (**SEQ ID NO:1**)) was targeted against the branch point sequence in intron 1 of ß-globin pre-mRNA. The 2'-O-methyl oligonucleotides were selected for this and subsequent experiments since they are resistant to nucleases and form stable hybrids with RNA that are not degraded by RNase H (H. Inoue et al., *Nucleic Acids Res.* **15**, 6131 (1987); H. Inoue et al., *FEBS Lett.* **215**, 327 (1987); B. Sproat et al., *Nucleic Acids Res.* **17**, 3373 (1989)). Degradation by RNase H, seen for example when antisense oligodeoxynucleotides or their phosphorothioate derivatives are used, would destroy the substrate pre-mRNA and prevent any splicing.

Figure 2 shows the reversal of aberrant splicing by oligonucleotide 1 directed against the normal branch point in intron 1 of ß-globin pre-mRNA. Splicing of P^{32} labeled $ß^{110}$ pre-mRNA (approximately 10^5 cpm per reaction, 25 fmoles) was carried out *in vitro* in HeLa cell nuclear extract for 2 hours, essentially as described (A. Krainer et al., *Cell* **36**, 993 (1984); Z. Dominski and R. Kole, *Mol. Cell. Biol.* **12**, 2108 (1992)) except that the volume of the reaction was doubled to 50 µl. Reaction products were analyzed on an 8% polyacrylamide sequencing gel and visualized by autoradiography. The structure of the products and intermediates is depicted on the right, their size in nucleotides is shown on the left. An asterisk denotes aberrant mobility of lariat-containing intermediates. Lane 1, splicing of control HBΔ6 pre-mRNA. Lane 2, splicing of $ß^{110}$ pre-mRNA. Lanes 3-8, splicing of $ß^{110}$ pre-mRNA in the presence of increasing amounts (indicated at the top of the figure) of oligonucleotide 1. Lane 9, splicing of $ß^{110}$ pre-mRNA in the presence of

oligonucleotide 3, targeted to a sequence in intron 2 of ß-globin pre-mRNA.

Analysis of these data shows that in the control reaction without the oligonucleotide (**Fig. 2**, lane 2), the ratio of the incorrectly to correctly spliced products is approximately 9:1. Addition of oligonucleotide 1 at concentrations 0.01 to 1.0 μg per reaction (0.05-5 μM) causes dose dependent inhibition of aberrant splicing and induction of the correct splicing of the substrate (**Fig. 2**, lanes 3-6). At 1.0 μg of the oligonucleotide the ratio of spliced products is reversed to 1:5. The effect of the oligonucleotide is sequence specific since addition of 1 μg of an oligonucleotide targeted against the cryptic 3' splice site in the second intron of the ß-globin gene (oligonucleotide 3, (**SEQ ID NO:3**); see also below) does not affect the original ratio of the spliced products (**Fig. 2**, lane 9). At 2.0 and 4.0 μg of oligonucleotide 1, splicing at both splice sites is inhibited and a 243-mer RNA fragment is generated (**Fig. 2**, lanes 7-8). This fragment accumulates only under splicing conditions, i.e. in the presence of ATP and other components of the splicing mixture, and most likely represents a product of cleavage at the site of the oligonucleotide's binding by an ATP dependent nuclease.

The aberrant 3' splice site generated by the $ß^{110}$ mutation also appears to be a target for reversal of aberrant splicing by an antisense oligonucleotide. Blocking of this sequence should be the simplest way of forcing the splicing machinery to use the original 3' splice site at the end of the intron. However, a 14-mer (oligo 2, (**SEQ ID NO:2**)) directed against the aberrant splice site was not effective; at increasing concentrations of the oligonucleotide accumulation of both spliced products was inhibited, the correct one being inhibited somewhat more efficiently (**Fig. 3**,

lanes 2-5). Splicing was carried out under the same conditions as described in connection with Fig. 2. Interestingly, the first step of the splicing reaction, cleavage at the 5' splice site and formation of the lariat-exon intermediate, seems to be less affected by oligo 2 than the formation of the final spliced product. This is shown by the presence of these intermediates even when 1 or 2 μg of the oligonucleotide were added to the splicing reaction (**Fig.** 3, lanes 5-6). At 4 μg per reaction cleavage at the 5' splice site is inhibited (**Fig.** 3, lane 7).

The different effects of oligo 1 and oligo 2 reflect complex interactions among the oligonucleotides, the numerous splicing factors and sequence elements located in the stretch of 37 nucleotides between the normal branch point and the correct 3' splice site. Clearly, oligonucleotide 1, hybridized to the normal branch point at the 5' end of this region, prevents binding of the splicing factors to this sequence forcing them to select the cryptic branch point downstream. This leads to inhibition of aberrant and induction of correct splicing of β^{110} pre-mRNA. In contrast, hybridization of oligo 2 to its centrally located target sequence may hinder binding of a large number of splicing factors that assemble in this region and prevent any splicing. Note also that this oligonucleotide blocks a significant portion of the polypyrimidine tract that is essential for splicing to both the aberrant and the correct 3' splice sites. This is an alternative explanation why this oligonucleotide failed to restore the correct splicing pathway.

EXAMPLE 4

Reversal of Aberrant Splicing by Antisense Oligonucleotides Against the 5' and 3' Splice Sites of Human β-Globin Intron 2

Whether an aberrant 3' splice site can nevertheless be used as a target for reversal of incorrect splicing was further tested on pre-mRNA carrying a T to G mutation at position 705 of the second intron of human ß-globin gene. This mutation ($IVS2^{705}$), found in Mediterranean thalassemia patients, creates an additional, aberrant 5' splice site 145 nucleotides upstream from the normal 3' splice site (C. Dobkin and A. Bank, *J. Biol. Chem.* **260**, 16332 (1985)). During splicing, a cryptic 3' splice site is activated at position 579 of the intron resulting in the removal of nucleotides 1-578 and 706-850 as separate introns and incorporation of the remaining portion of the intron into the spliced product (**Fig. 1**). In this RNA the distances between each of the sequence elements involved in splicing exceed 100 nucleotides and no steric hindrance effects by the oligonucleotide should be expected.

The reversal of aberrant splicing of $IVS2^{705}$ pre-mRNA by oligonucleotide 3 (**SEQ ID NO:3**) directed against the cryptic 3' splice site and oligonucleotide 4 (**SEQ ID NO:4**) directed against the aberrant 5' splice site in intron 2 of the $IVS2^{705}$ pre-mRNA is shown in **Figure 4**. The conditions of the splicing reaction were the same as described in connection with Fig. 2 above, except that before use the RNA transcript was purified by electrophoresis on a 6% sequencing gel. Lane 1, input RNA. Lanes 2 and 3, splicing of control transcripts (indicated at the top of the figure). Lanes 4-8 and 9-13, splicing of $IVS2^{705}$ pre-mRNA in the presence of oligonucleotide 3 and oligonucleotide 4, respectively. The amounts of the oligonucleotides in

the reaction are indicated at the top. "?" on the left indicates apparent degradation product.

The control transcript containing the second intron of normal ß-globin pre-mRNA is spliced efficiently (**Fig. 4**, lane 2) generating the expected intermediates (the 5′ exon and the large lariats) and the correctly spliced product, 451 nucleotides in length. Splicing of $IVS2^{705}$ pre-mRNA is also efficient and yields an additional spliced product 577 nucleotide long and an expected 348-mer intermediate, resulting from the aberrant splicing pathway caused by the mutation (**Fig.4** , lane 3). The 1:2 ratio of correctly to incorrectly spliced RNAs is similar to that observed previously *in vivo*. Oligonucleotide 3 (**Fig.** 1) targeted at the activated cryptic 3′ splice site at nucleotide 579 is very active, inducing dose dependent reversal of splicing to the correct splicing pathway (**Fig. 4**, lanes 4-8). At 0.1 and 0.4 μg of the oligonucleotide per reaction the reversal is virtually complete. Correct splicing is also obtained at similar concentrations of oligonucleotide 4 (**Fig. 1**) targeted against the aberrant 5′ splice site created by the mutation at nucleotide 705 of the second intron (**Fig. 4**, lanes 9-13). At 1 and 2 μg per reaction, either oligonucleotide had no additional effects; at 4 μg per reaction (20 μM) all splicing is inhibited (not shown). Additional bands, including a strong band marked by "?" in a figure are most likely due to nuclease degradation of the long (1301 nucleotides) pre-mRNA.

These results show that the cryptic 3′ splice site as well as the mutated 5′ splice site provide suitable targets for specific reversal of aberrant splicing. Similar effects of oligonucleotides 3 and 4 suggest that there are no major differences in their accessibilities to the target splice sites. Both oligonucleotides are approximately 10 times more effective than oligonucleotide 1 used in the

experiments shown in **Fig. 2**. This higher efficiency may be due to several factors. Oligonucleotides 3 and 4 are three nucleotides longer than oligonucleotide 1 and may form more stable hybrids with RNA. They block aberrant splice sites, allowing the splicing machinery to use the correct splice sites and, presumably, the correct branch point. In contrast, in β^{110} pre-mRNA oligonucleotide 1 forces the splicing machinery to use a suboptimal cryptic branch point sequence, which may result in relatively inefficient generation of correctly spliced mRNA. In experiments shown in **Fig. 4** the long input pre-mRNA is barely detectable after 2 hours of the reaction, suggesting its instability. Thus, although the molar concentrations of the oligonucleotides were essentially the same as in previous experiments they may have been in greater excess over the substrate pre-mRNA.

In the experiments presented above the oligonucleotides were added simultaneously with the other components of the splicing reaction. Prehybridization of the oligonucleotides with the pre-mRNA did not increase their efficiency and oligonucleotides added 15 minutes after the start of the reaction, i.e. after splicing complexes had a chance to form (B. Ruskin and M. Green, *Cell* **43**, 131 (1985)), were almost as effective (data not shown). These results indicate that oligonucleotides containing the 2'-O-methyl modification are able to compete effectively for their target sequences with the splicing factors. The high activity of these compounds is most likely due to their strong hybridization to RNA.

EXAMPLE 5

Reversal of Aberrant Splicing With an Antisense Oligonucleotide Which Blocks the Cryptic 3' Splice Site the IVS1-5 and IVS1-6

This experiment is carried out essentially as described above, except that the thalassemic mutations are the IVS1-5 and IVS1-6 mutations, in which the authentic 5' splice site of IVS1 is mutated. Aberrant splicing resulting in thalassemia is apparently due to the fact that mutations IVS1-5 and IVS1-6 weaken the 5' splice site and allow the cryptic splice site located 16 nucleotides upstream to successfully compete for the splicing factors. In this experiement we test whether an oligonucleotide antisense to the cryptic splice site may revert aberrant splicing back to the mutated 5' splice site and restore correct splicing in spite of the mutations, since splice sites similar to the mutated ones appear functional in other pre-mRNAs. The oligonucleotide employed is oligo 6 (**SEQ ID NO:6**), a 2-O-methyl-ribooligonucleotide produced as described in Example 2 above.

EXAMPLE 6

Reversal of Aberrant Splicing With an Antisense Oligonucleotide Which Blocks the Aberrant 5' Splice Site of the $IVS2^{654}$ Mutation

These experiments are carried out essentially as described above, except that the human β-globin pre-mRNA containing the $IVS2^{654}$ mutation is employed, and oligo 5 (**SEQ ID NO:5**) is employed.

The $IVS2^{654}$ mutation, frequently identified in thalassemic individuals of Chinese origin, affects splicing by creating an additional 5' splice site at nucleotide 653 and activating the common cryptic 3' splice site at nucleotide 579 of intron 2. The efficiency of aberrant splicing of $IVS2^{654}$ pre-mRNA is higher than that for $IVS2^{705}$ pre-mRNA and only small amounts of correctly spliced product, relative to the

aberrant one, are detectable during splicing *in vitro*. In spite of the high efficiency of aberrant splicing, oligo 3, targeted against the cryptic 3' splice site, as well as oligo 5, targeted against the aberrant 5' splice site, restored correct splicing efficiently at concentrations similar to those described above. At 2 μM concentration of either oligonucleotide the correctly spliced product accumulates and the aberrant product is virtually undetectable (data not shown).

EXAMPLE 7

Reversal of Aberrant Splicing by Antisense Oligonucleotide Which Blocks the Human β-Globin Intron 1 Branch Point

This experiment is carried out essentially as described above to restore correct splicing in β-110 mutant pre-mRNA, except the oligonucleotide binds to to a sequence located just upstream from the native branch point sequence of intron 1 of β-globin gene (nucleotides 75-88). The sequence of the oligonucleotide is: CCCAAAGACUAUCC (**SEQ ID NO:7**). Correct splicing is restored.

EXAMPLE 8

Construction of Cell Lines Expressing Thalassemic Human β-Globin pre-mRNA

A series of stable cell lines are constructed by transfecting HeLa cells and CHO cells with thalassemic globin genes cloned under the cytomegalovirus (CMV) immediate early promoter. The genes include IVS1-110, IVS2-654 and IVS1-5 mutation.

Stable cell lines are obtained in accordance with standard techniques. *See, e.g.*, Current Protocols in Molecular Biology (P. Ausubel. et al. eds. 1987). Briefly, cells are cotransfected with plasmids carrying thalassemic globin genes under the CMV promoter and plasmids carrying the neomycin resistance gene as a selectable marker (pSV2neo). Transfection is either by electroporation, (Z. Dominski and R. Kole, *Mol. Cel.*

Biol. **11**: 6075-6083 (1991); Z. Dominski and R. Kole, *Mol. Cell. Biol.* **12**: 2108-2114 (1992)), or by the calcium phosphate method. Cells are plated and after 24-48 hours challenged with selective medium containing G418. Surviving colonies are expanded and assayed for expression of thalassemic globin mRNA as follows.

Total RNA is isolated from approximately 10^5 cells using a commercial Tri-Reagent (Molecular Research Center, Cincinnati, OH) following manufacturer's protocol. This method allows for easy processing of a large number of small samples and gives high yields of good quality RNA. The splicing patterns are analyzed by RT-PCR using rTth polymerase and following the manufacturers protocol (Perkin Elmer). No more than 1-5% of isolated RNA is required for detection of spliced RNA in transiently transfected cells, thus the method is sufficiently sensitive for easy detection in stable cell lines. The reverse transcriptase step is carried out with a 3' primer that hybridizes to the aberrant sequences in thalassemic mRNA and spans the splice junction. This assures that the contaminating DNA and normal globin RNA are not detected and do not interfere with the assay. The cloned cell lines that express thalassemic pre-mRNA are used for treatment with antisense 2'-O-methyl-oligonucleotides as described below.

EXAMPLE 9

Administration of Antisense Oligonucleotides In Cell Culture

Cells produced in Example 8 above are grown in 24 well culture dishes containing 200 μl of media per well. $2x10^4$ cells are seeded per well and when attached they are treated with 200 μl of media containing up to 50 μM concentration of antisense oligonucleotides. Cells are cultured up to 4 days in the presence of the oligonucleotide before reaching confluence ($2-3x10^5$ cells). Since 2'-O-methyl

oligonucleotides are very stable in serum containing media, medium is changed no more than every two days. The 50 μM (40 μg per well) concentration of the oligonucleotide represents 100 fold excess over that required to elicit efficient restoration of splicing *in vitro*. Even at this concentration a single oligonucleotide synthesis at 1 μmole scale, yielding 1-1.6 mg of the oligonucleotide, provides sufficient material for 25 to 40 samples.

In an alternative approach cells are pretreated with Lipofectin™ reagent (DOTMA, from BRL) at a concentration of 10 μg/ml before addition of oligonucleotides, in accordance with known techniques. (C. Bennett et al., *Mol. Pharm.* **41**: 1023-1033 (1992)).

After treatment total RNA is isolated as above and assayed for the presence of correctly spliced mRNA by RT-PCR. Amplification of primers is carried out in the presence of alpha-P32 labeled ATP to increase sensitivity of detection and reduce the number of cycles to 15.

The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

EXAMPLE 10

Liposome Transfection

A HeLa based cell line stably transfected with the human β-globin clone carrying an IVS2-654 thalassemic mutation was used to carry out the following experiments. One subclone of the ISV2-654 cell line expresses predominantly aberrantly spliced β-globin mRNA and small amounts of the correctly spliced species. A second subclone, termed ISV2-654*, expresses aberrantly and correctly spliced β-globin mRNAs in approximately a 1:1 ratio. Approximately 10^5 of IVS2-654 cells grown in monolayer were treated with

3 μM of the 2'-O-methyl-ribooligonucleotide (17-mer) antisense to the 3' cryptic splice site [oligo 3; CAUUAUUGCCCUGAAAG; (**SEQ ID NO:3**)] in the presence of 20 μg/ml of LIPOFECTIN™ liposome transfection reagent (BRL) in accordance with the manufacturer's specifications for 5 hours in OPTI-MEM medium without serum. After incubation, the medium was removed and the cells returned to the normal growth medium (MEM + 10% fetal calf serum). Untreated cells and cells treated with lipofection alone were used as controls. After overnight growth, total cell RNA was isolated using Tri-Reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocol. The RNA was quantitated by absorbance at 260 nm.

Analysis of splicing was carried out by reverse-transcriptase-PCR (RT-PCR). An equal amount of RNA from each sample was assayed using a rTth RT-PCR kit (Cetus-Perkin Elmer). The manufacturer's protocol was followed with the exception that 1 μCi of α-P32 labelled dATP was added to the RT-PCR reaction. PCR products were analyzed on 8% nondenaturing polyacrylamide gel. An oligonucleotide hybridizing to the second exon of human β-globin gene was used as a common primer. Primer specific for aberrant splicing (primer A) spanned the aberrant splice junction whereas primer for correct splicing (primer C) spanned the correct splice junction (**Figure 5**). RT-PCR was carried out with primer A in lanes 1, 3 and 5 and with primer C in lanes 2, 4 and 6. The amount of PCR product loaded in lanes 1, 3 and 5 was 1/5 of that in lanes 2, 4 and 6. Lanes 1 and 2 show splicing of pre-mRNA from cells treated with LIPOFECTIN™ reagent and 3 μM 2'-O-methyl-oligoribo-nucleotide; lanes 3 and 4 show splicing of pre-mRNA from cells treated with LIPOFECTIN™ reagent alone; and lanes 5 and 6 show splicing of pre-mRNA from untreated cells. Note the visually detectable increase in correct splicing for cells treated with the

oligonucleotide in the presence of the LIPOFECTIN™ reagent in lane 2.

EXAMPLE 11

Adenovirus Mediated Transfer

Approximately 10^5 of ISV2-654* cells grown in monolayer overnight were treated with 5 and 20 μM 2'-O-methyl-ribooligonucleotide (17-mer) antisense to the 3' cryptic splice site (**SEQ ID NO:3**) in the presence of 10^6 particles of replication deficient adenovirus (strain dl-312, a gift from Dr. Hu of the University of North Carolina at Chapel Hill). The virus and oligonucleotide were preincubated for 30 minutes in OPTI-MEM, then added to the cell culture, and the incubation continued for 2 hours. The medium was aspirated and replaced with a normal growth medium. After overnight growth, the total RNA was isolated as described above. The RT-PCR reaction was carried out as above with the exception that the primers hybridized to the second and third exons of the human β-globin gene respectively. Analysis of the products was as given in Example 10 above with the exception that equal amounts of PCR products, were loaded on the gel. Results are illustrated in **Figure 6**. Lane 1 showed splicing of untreated cells; and lanes 2 and 3 showed splicing of cells treated with 5 and 20 μM oligonucleotide, respectively, in the presence of adenovirus. Note the visually detectable increase in correct splicing, along with a corresponding decrease in aberrant splicing and in a dose-dependent manner, for the 5 and 20 μM oligo-treated cells.

EXAMPLE 12

Electroporation

Approximately 10^5 IVS2-654* cells grown overnight in monolayer were trypsinized and transferred in 0.5 ml of OPTI-MEM to a 10 mm electroporation cuvette. 5 or 50 μM 2'-O-methyl- ribooligonucleotide

antisense to the aberrant 5' splice site [oligo 4; (CCUCUUACCUCAGUUAC) (**SEQ ID NO:4**)] was added per sample and the mixture was electroporated with a 1500V pulse using an University of Wisconsin Electronics Laboratory electroporator set at 0.75 μF. After electroporation, cells were resuspended in the normal growth medium and grown in monolayer overnight. Total RNA was isolated as described above and analyzed by RT-PCR as described in Example 11 above.

Results are illustrated in **Figure 7**. Note a visually detectable decrease in aberrant splicing, along with a corresponding increase in correct splicing, particularly where 50 μM of oligo is delivered.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Dominski, Zbigniew T.

(ii) TITLE OF INVENTION: Antisense Oligonucleotides Which Combat Aberrant Splicing and Methods of Using the Same

(iii) NUMBER OF SEQUENCES: 7

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) TELEX: 575102

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CAUUAUUGCC CUGAAAG 17

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCUCUUACCU CAGUUAC 17

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCUAUUACCU UAACCCAG 18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GCCUGACCAC CAAC 14

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA (genomic)

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCCAAAGACU AUCC 14

THAT WHICH IS CLAIMED IS:

1. A method of combatting aberrant splicing in a pre-mRNA molecule containing a mutation,

wherein said pre-mRNA molecule contains a first set of splice elements defining a native intron which is removed by splicing when said mutation is absent to produce a first mRNA molecule encoding a native protein;

and wherein said pre-mRNA further contains a second set of splice elements induced by said mutation and defining an aberrant intron different from said native intron, which aberrant intron is removed by splicing when said mutation is present to produce an aberrant second mRNA molecule different from said first mRNA molecule;

said method comprising:

hybridizing an antisense oligonucleotide to said pre-mRNA molecule to create a duplex molecule under conditions which permit splicing,

wherein said antisense oligonucleotide does not activate RNase H;

and wherein said oligonucleotide blocks a member of said aberrant second set of splice elements;

so that said native intron is removed by splicing and said first mRNA molecule encoding a protein is produced.

2. A method according to claim 1, wherein said antisense oligonucleotide blocks a mutated splice element.

3. A method according to claim 1, wherein said antisense oligonucleotide blocks a native splice element.

4. A method according to claim 1, wherein said antisense oligonucleotide blocks a cryptic splice element.

5. A method according to claim 1, wherein said antisense oligonucleotide blocks a 5' splice site.

6. A method according to claim 1, wherein said antisense oligonucleotide blocks a 3' splice site.

7. A method according to claim 1, wherein said antisense oligonucleotide blocks a branch point.

8. A method according to claim 1, wherein said hybridizing step is carried out in a cell, and wherein said first mRNA is translated into said native protein.

9. A method according to claim 1, wherein said native protein is β-globin.

10. A method according to claim 1, wherein said native protein is human β-globin.

11. A method according to claim 1, wherein said antisense oligonucleotide is from 8 to 50 nucleotides in length.

12. A method according to claim 1, wherein said antisense oligonucleotide contains a modified internucleotide bridging phosphate residue selected from the group consisting of methyl phosphonates, methyl phosphonothioates, phosphoromorpholidates, phosphoropiperazidates, and phosphoramidates.

13. A method according to claim 1, wherein said antisense oligonucleotide contains a nucleotide having a loweralkyl substituent at the 2' position thereof.

14. A method of upregulating expression of a native protein in a cell containing a DNA encoding said native protein, which DNA contains a mutation which causes downregulation of said native protein by aberrant splicing thereof,

wherein said DNA encodes a pre-mRNA;

wherein said pre-mRNA contains a first set of splice elements defining a native intron which is removed by splicing when said mutation is absent to produce a first mRNA encoding said native protein;

and wherein said pre-mRNA further contains a second set of splice elements induced by said mutation and defining an aberrant intron different from said native intron, which aberrant intron is removed by splicing when said mutation is present to produce an aberrant second mRNA different from said first mRNA;

said method comprising:

administering to said cell an antisense oligonucleotide which hybridizes to said pre-mRNA to create a duplex thereof under conditions which permit splicing,

wherein said antisense oligonucleotide does not activate RNase H;

and wherein said antisense oligonucleotide blocks a member of said aberrant second set of splice elements;

so that said native intron is removed by splicing and said native protein is produced.

15. A method according to claim 14, wherein said cell is a eukaryotic cell selected from the group consisting of yeast, insect, and mammalian cells.

16. An antisense oligonucleotide useful for combatting aberrant splicing in a pre-mRNA molecule containing a mutation,

wherein said pre-mRNA molecule contains a first set of splice elements defining a native intron which is removed by splicing when said mutation is absent to produce a first mRNA molecule encoding a native protein;

and wherein said pre-mRNA further contains a second set of splice elements induced by said mutation and defining an aberrant intron different from said native intron, which aberrant intron is preferentially removed by splicing when said mutation is present to produce an aberrant second mRNA molecule different from said first mRNA molecule;

said antisense oligonucleotide comprising an oligonucleotide which:

(i) hybridizes to said pre-mRNA to form a duplex molecule;

(ii) does not activate RNase H; and

(iii) blocks a member of said aberrant second set of splice elements.

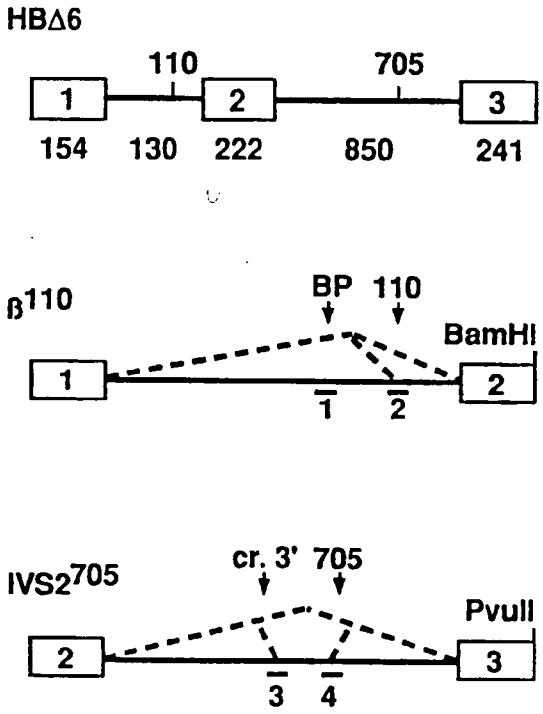
17. An antisense oligonucleotide according to claim 16, wherein said oligonucleotide is 8 to 50 nucleotides in length.

18. An antisense oligonucleotide according to claim 16, wherein said oligonucleotide contains a modified internucleotide bridging phosphate residue selected from the group consisting of methyl phosphonates, methyl phosphonothioates, phosphoromorpholidates, phosphoropiperazidates, and phosphoramidates.

19. An antisense oligonucleotide according to claim 16, wherein said antisense oligonucleotide contains a nucleotide having a loweralkyl substituent at the 2' position thereof.

20. An antisense oligonucleotide according to claim 16 in an aqueous physiologically acceptable carrier solution.

21. An antisense oligonucleotide according to claim 16 in a lipid vesicle.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3
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850
241
β110
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110
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705
PvuII
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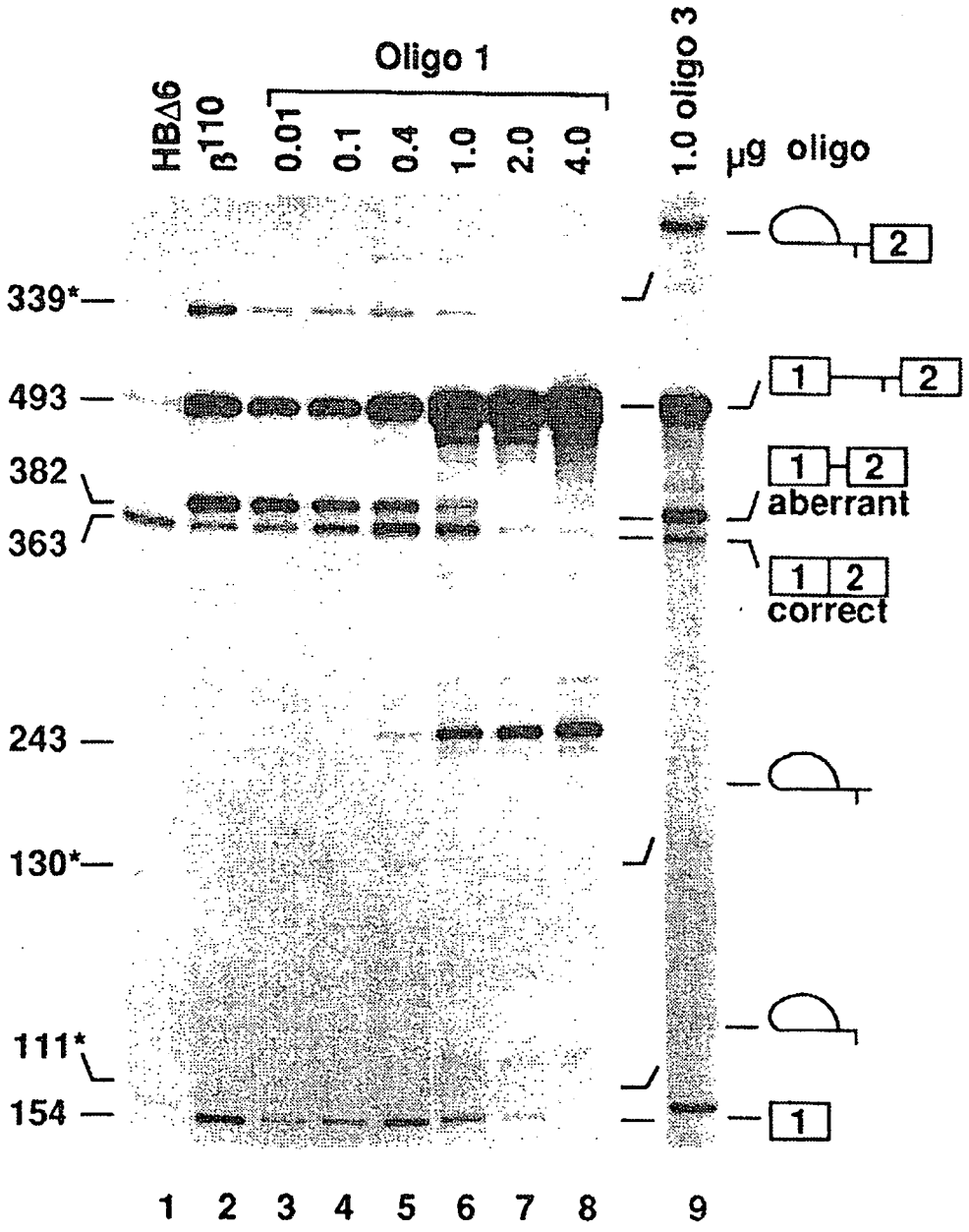
3
4

FIG. 1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β110
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0.1
0.4
1.0
2.0
4.0
1.0 oligo 3
μg oligo
339*
493
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aberrant
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correct
1
1 2 3 4 5 6 7 8 9

FIG. 2

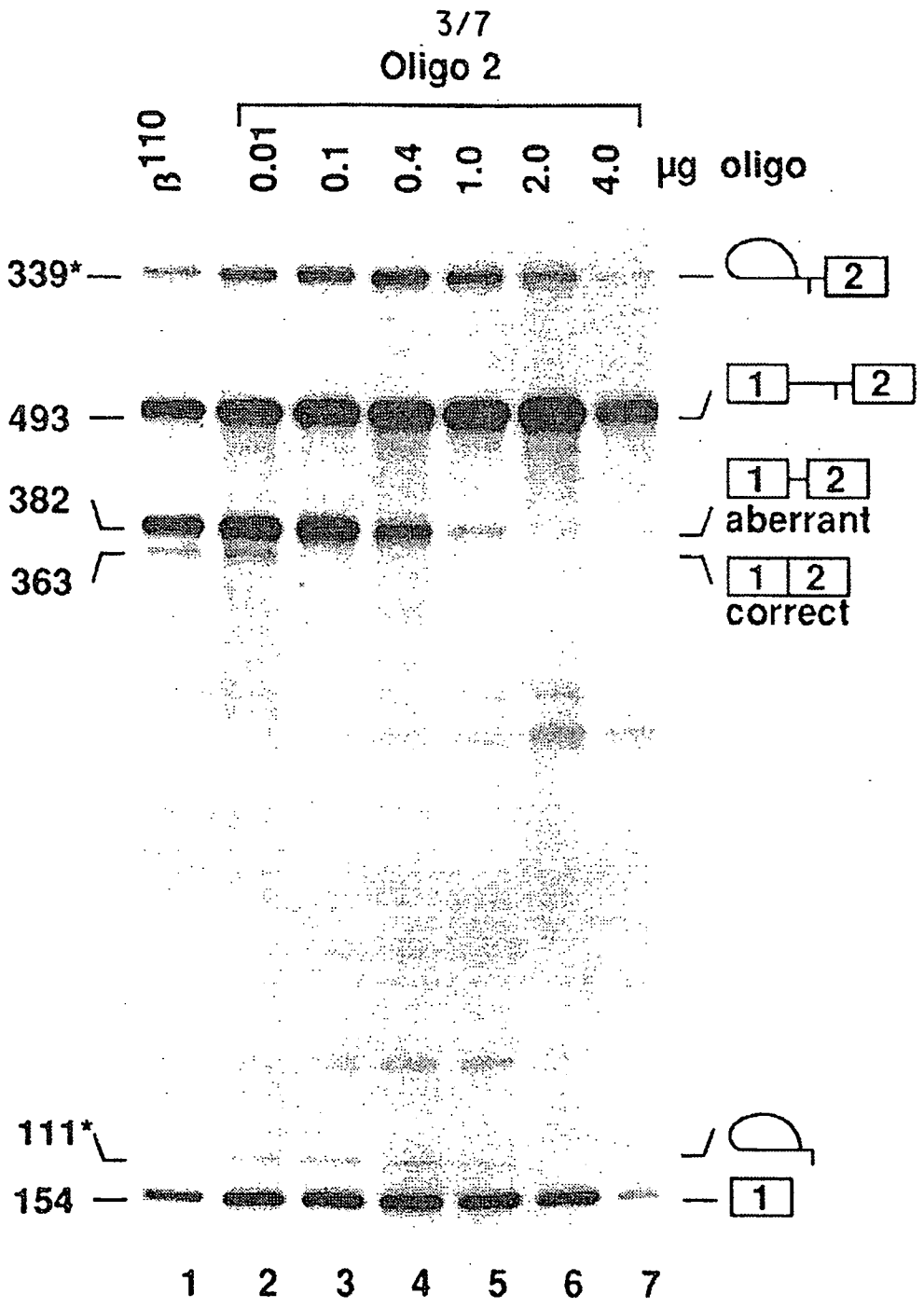
3/7
Oligo 2
β110
0.01
0.1
0.4
1.0
2.0
4.0
µg oligo
339*
493
382
363
111*
154
1
2
aberrant
correct
1 2 3 4 5 6 7

FIG. 3

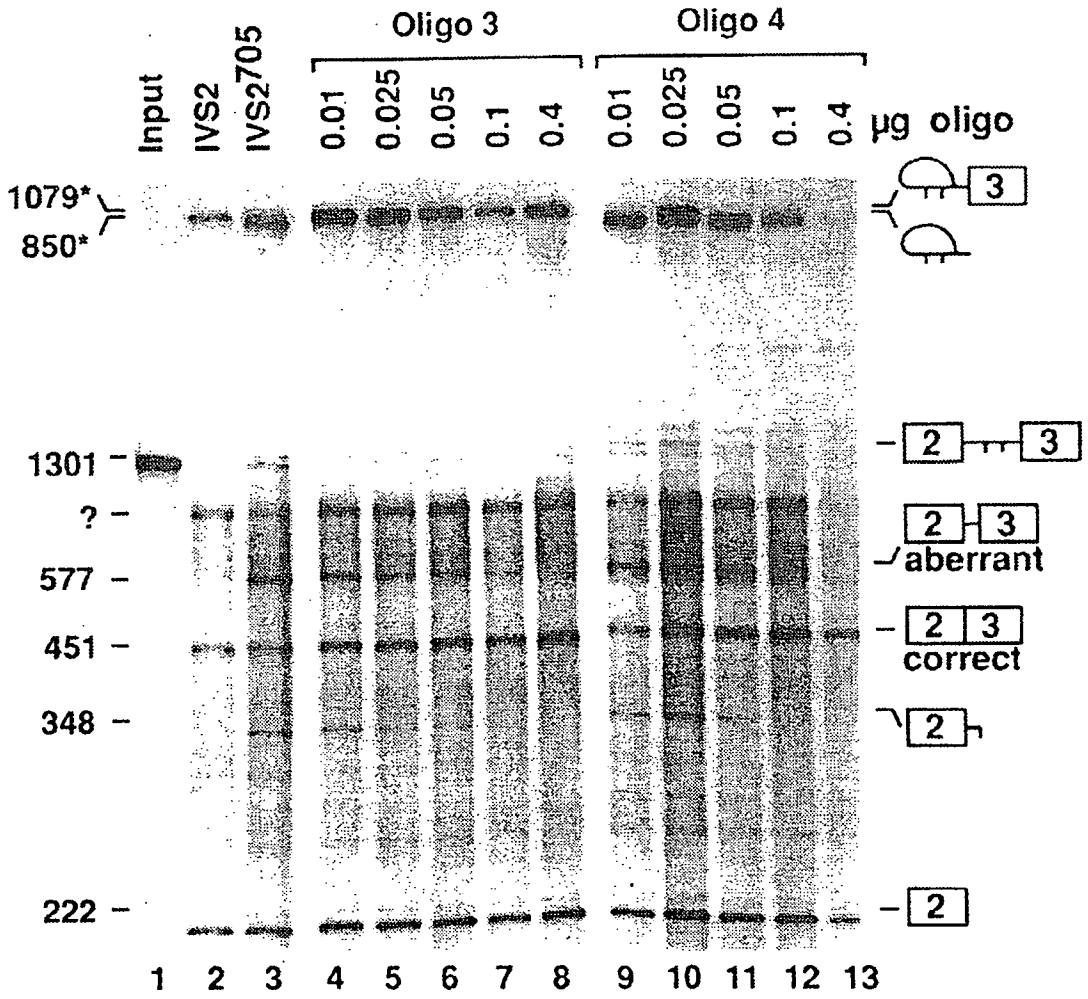
Oligo 3
Oligo 4
Input
IVS2
IVS2705
0.01
0.025
0.05
0.1
0.4
0.01
0.025
0.05
0.1
0.4
µg oligo
1079*
850*
3
1301
?
577
451
348
222
2 3
2 3
aberrant
2 3
correct
2
2
1 2 3 4 5 6 7 8 9 10 11 12 13

FIG. 4

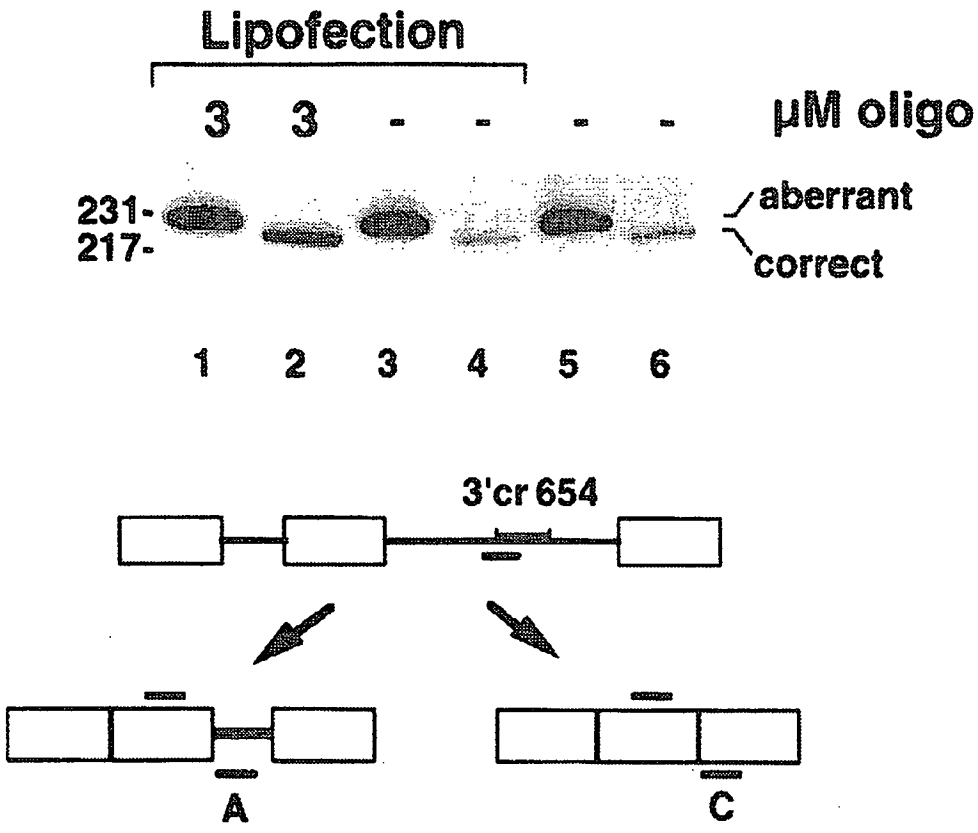
Lipofection
3 3 - - - - µM oligo
231-
217-
aberrant
correct
1 2 3 4 5 6
3'cr 654
A
C

FIG. 5

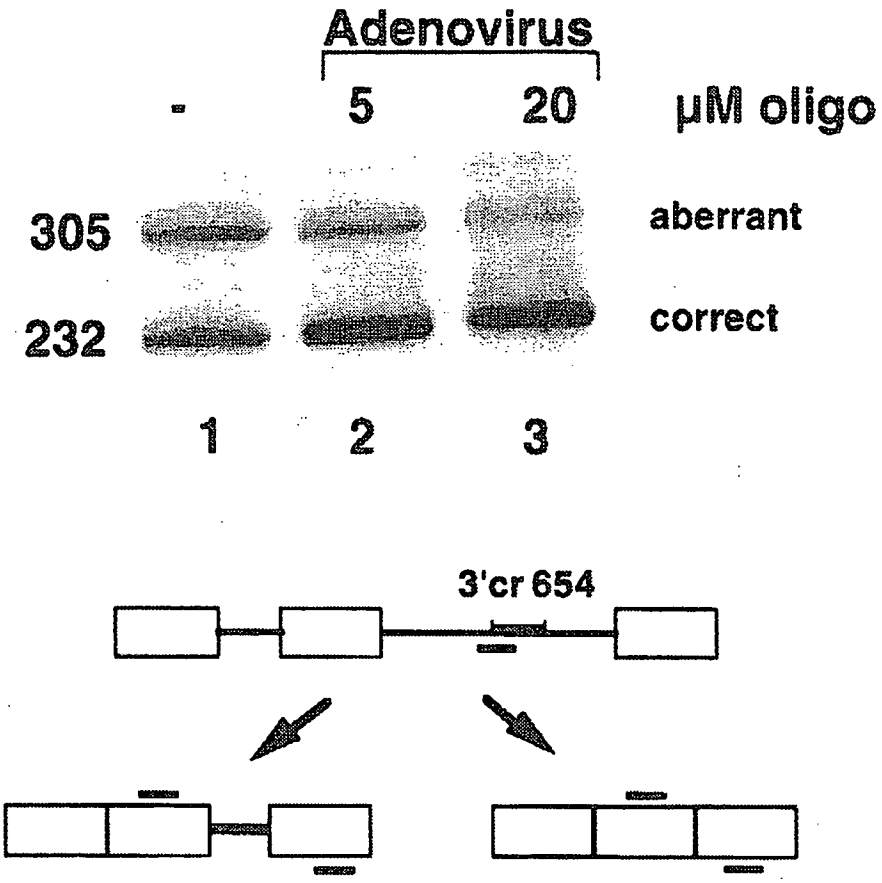
Adenovirus
-
5
20
μM oligo
305
232
aberrant
correct
1
2
3
3'cr 654

FIG. 6

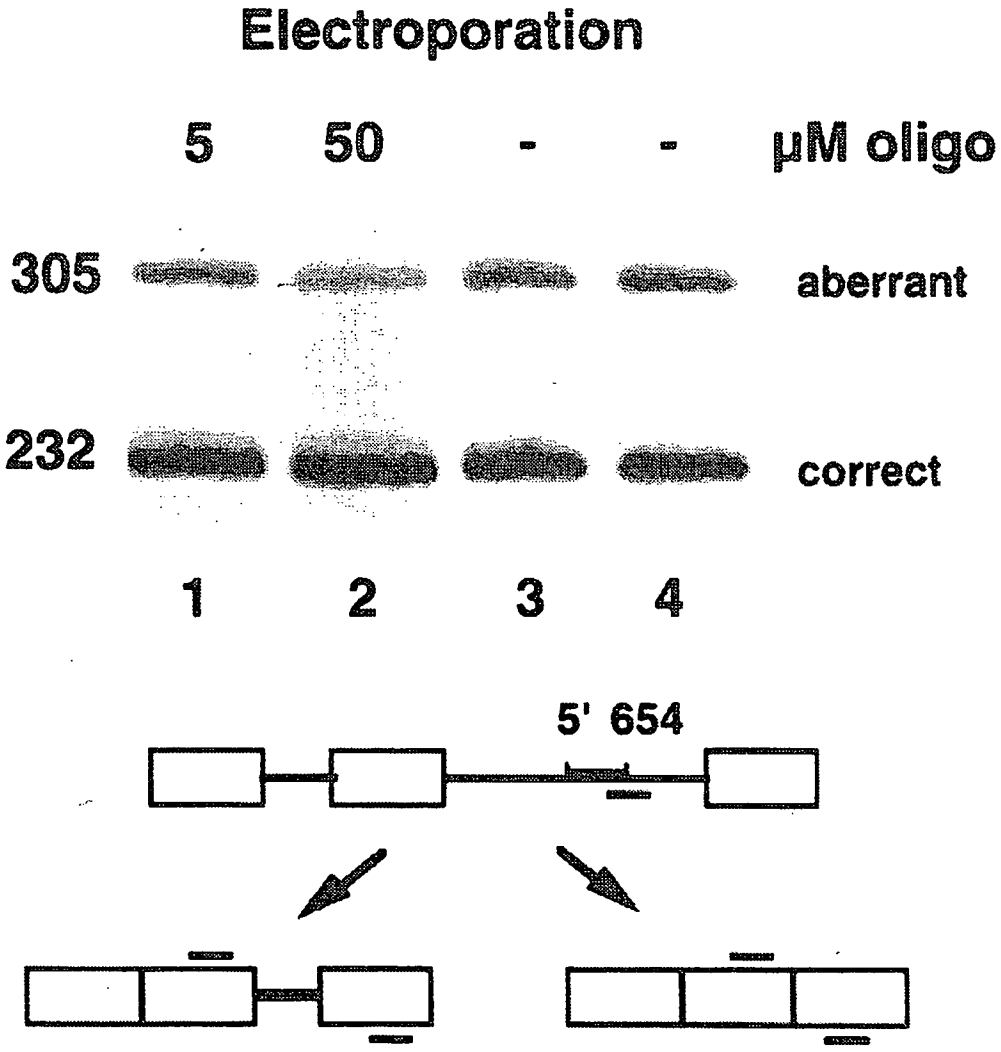
Electroporation
5
50
-
-
µM oligo
305
aberrant
232
correct
1
2
3
4
5' 654

FIG. 7

INTERNATIONAL SEARCH REPORT

Interr nal Application No
PCT/US 94/05181

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5 C12N15/11 A61K31/70 A61K48/00 //C12N15/67

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.13D, 1989 FURDON, P. & KOLE, R. 'Inhibition of in vitro pre-mRNA splicing by antisense deoxyoligonucleotide analogues'	1-5, 8-10,12, 14-16,18
Y	see abstract K210 ---	1-21
Y	ADVANCED DRUG DELIVERY REVIEWS, vol.6, no.3, June 1991 pages 271 - 286 KOLE, R. ET AL. 'Pre-mRNA splicing as a target for antisense oligonucleotides' see whole chapter III --- -/--	1-19

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 October 1994	07.10.94

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Andres, S

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MOLECULAR PHARMACOLOGY, vol.41, no.6, June 1992 pages 1023 - 1033 BENNETT, F. ET AL. 'Cationic lipids enhance cellular uptake and activity of phosphorothioate antisense oligonucleotides' cited in the application see the whole document	20,21
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol.179, no.3, 30 September 1991, DULUTH, MINNESOTA US pages 1593 - 1599 VOLLOCH, V. & AL. 'Inhibition of pre-mRNA splicing by antisense RNA in vitro: effect of RNA containing sequences complementary to exons' see the whole document	1,3,5,6, 9,11,17
A	GENES & DEVELOPMENT, vol.3, no.10, October 1989, COLD SPRING HARBOR, USA pages 1545 - 1552 ZHUANG, Y. & WEINER, A. 'A compensatory base change in human U2 snRNA can suppress a branch site mutation' cited in the application see Results	4,7
A	EMBO JOURNAL, vol.7, no.8, 1988, EYNSHAM, OXFORD GB pages 2523 - 2532 MUNROE, S. 'Antisense RNA inhibits splicing of pre-mRNA in vitro'	
P,X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol.90, September 1993, WASHINGTON US pages 8673 - 8677 DOMINSKI, Z. & KOLE, R. 'Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides' see the whole document	1-19

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/05181

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 1-15 (as far as in vivo methods are concerned) are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.